Epratuzumab, a Humanized Monoclonal Antibody Targeting CD22: Characterization of *in Vitro* Properties[1]

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Abstract

Purpose: **Epratuzumab is a novel humanized antihuman CD22 IgG1 antibody that has recently shown promising clinical activity, both as a single agent and in combination with rituximab, in patients with non-Hodgkin's lymphomas (NHL). In an attempt to better understand the mode of action of epratuzumab, the antibody was tested *in vitro* in a variety of cell-based assays similar to those used to evaluate the biological activity of other therapeutic monoclonal antibodies, including rituximab. In this report, we present epratuzumab activities as they relate to binding, signaling, and internalization of the receptor CD22.**

Methods: **Chinese hamster ovary-expressed CD22 extracellular domain was used to measure epratuzumab affinity on Biacore. CD22 receptor density and internalization rate were measured indirectly using a monovalently labeled, noncompeting (with epratuzumab) anti-CD22 antibody on Burkitt lymphoma cell lines, primary B cells derived from fresh tonsils, and B cells separated from peripheral blood samples obtained from patients with chronic lymphocytic leukemia or healthy volunteers. Epratuzumab-induced CD22 phosphorylation was measured by immunoprecipitation/Western blot and compared with that induced by anti-IgM stimulation.**

Results: **Epratuzumab binds to CD22-extracellular domain, with an affinity of K_D = 0.7 nM. Binding of epratuzumab to B cell lines, or primary B cells from healthy individuals and patients with NHL, results in rapid internalization of the CD22/antibody complex. Internalization appears to be faster at early time points in cell lines than in primary B cells and NHL patient-derived B cells, but the maximum internalization reached is comparable for all B cell populations after several hours of treatment and appears to reach saturation at antibody concentrations of 1–5 μg/ml. Finally, epratuzumab binding results in modest but significant CD22 phosphorylation.**

Conclusions: **Epratuzumab represents an excellent anti-CD22 ligating agent, highly efficacious in inducing CD22 internalization, and can induce phosphorylation. Although we cannot unequivocally demonstrate here that epratuzumab-induced internalization and signaling of CD22 directly contribute to its therapeutic efficacy, these properties are the fundamental characteristics of the target CD22 and its interaction with epratuzumab. Similar results were observed when epratuzumab was tested *in vitro* on Burkitt B cell lines as well as on primary normal B cells and neoplastic B cells separated from fresh peripheral blood samples from patients with chronic lymphocytic leukemia.**

Introduction

CD22 is a 135-kDa transmembrane sialoglycoprotein and a member of the immunoglobulin superfamily. Its expression is restricted to lymphocytes of the B cell lineage and is highly developmentally regulated: CD22 is present in the cytoplasm of pro- and pre-B cells and becomes detectable on the cell surface only at mature stages of B cell differentiation. Cell surface expression is lost during terminal differentiation into plasma cell and after B cell activation (1–3). CD22 is also expressed by the vast majority of B cell NHLs[3] (4). The CD22 molecule has multiple ligands because it binds to α2–6-linked sialic acid residues present on glycoproteins expressed by activated T and B cells, monocytes, neutrophils, erythrocytes, and activated endothelial cells (5). Although its function is not yet well understood, CD22 appears to be involved in the regulation of B cell activation through BCR signaling, (demonstrating both positive and negative roles *in vitro*) as well as in cell adhesion (6). *In vivo*, the important biological functions of this receptor have been demonstrated by genetic disruption of CD22. CD22-deficient mice have a shorter life span, a reduced number of mature B cells in the bone marrow and in circulation, and a chronic exaggerated antibody response to antigen and develop elevated levels of autoantibodies, suggesting a key role for CD22 in B cell development, survival, and function (1, 7–9).

Because the expression of CD22 is lineage restricted and, in most cases, is not lost during neoplastic transformation, it represents an attractive target for anti-NHL immunotherapeutic antibodies. Preclinical work with anti-CD22-"blocking" monoclonal antibodies (*i.e.*, monoclonal antibodies that prevent CD22

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[3] The abbreviations used are: NHL, non-Hodgkins lymphoma; BCR, B cell receptor; ECD, extracellular domain; PE, phycoerythrin; CLL, chronic lymphocytic leukemia.

binding to its natural ligands) suggests that modulation of receptor activity has a selective cytotoxic effect on receptor-positive tumor cells (10). Interestingly, the same ligand blocking anti-CD22 monoclonal antibody can trigger primary B cell proliferation, suggesting that the consequences of engaging CD22 may differ depending on the B cell stage of differentiation. In animal models, anti-CD22 monoclonal antibodies can mediate antitumor effects (11, 12). Unlike the B cell antigen CD20, CD22 internalizes rapidly upon antibody binding, making it an ideal target for delivery of radioisotopes or toxins to malignant cells via monoclonal antibody conjugates (11, 13–15).

LL2 is a mouse anti-CD22 monoclonal antibody (originally named EPB/2) generated against the Raji Burkitt's lymphoma cell line (16). LL2 is highly selective for B cell tumors but lacks reactivity with Hodgkin's disease, other solid tumors, or non-lymphoid tissues (17). A humanized version of the LL2 antibody, epratuzumab, was developed to minimize the potential for immunogenicity and enhancing effector interactions during its development as a diagnostic and immunotherapeutic (18). As a single agent, epratuzumab has shown preliminary evidence of antitumor activity in patients with recurrent NHL, producing responses, including durable complete responses (19). Objective responses were seen in 9 of 51 indolent NHL patients (17.6%; 3 complete responses, 6 partial responses) with a median duration of response of 47+ (range, 11–99+) weeks and median time to progression of 103+ (35–107+) weeks by Kaplan-Meier estimate. Of 52 aggressive NHL patients, 5 achieved objective responses (10%; 3 complete responses, 2 partial responses), with median duration of response 38+ (13 to 38+) weeks and median time to progression 35+ (23 to 35+) weeks. All responses occurred in the follicular NHL and diffuse large B cell histologies (19). Anti-CD22 agents are likely to have mechanism(s) of action distinct from those of other cytotoxic agents as well as from immunotherapies targeting other B cell antigens (*e.g.,* the anti-CD20 monoclonal antibody rituximab and anti-CD52 antibody alemtuzumab). Thus, they are theoretically good candidates for combination with other drugs in the treatment of B cell malignancies. Epratuzumab is currently being evaluated in combination with rituximab, and early results from an ongoing study suggest that the combination of the two antibodies is well tolerated and may result in improved clinical activity *versus* the single agents alone (20).

This paper reports the results of *in vitro* studies to further characterize the mechanism of action of epratuzumab by exploring its interaction with CD22.

Materials and Methods

Cell Lines and Reagents. The CD22-expressing human Burkitt's lymphoma cell lines, Daudi, Ramos, Namalwa, and Raji, were obtained from the American Type Culture Collection (Manassas, VA). The cell lines were grown in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FBS (HyClone, Logan, UT), 2 mM glutamine,100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cells were cultured at 37°C in a humidified 5% CO_2 incubator. $F(ab')_2$ fragments of goat antihuman IgG(Fc) were purchased from Rockland Immunochemicals, Gilbertsville, PA. Other reagents included: $F(ab')_2$ fragment of goat antihuman IgM, Fc_{5u} (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), sodium orthovanadate (Sigma-Aldrich, St. Louis, MO), complete protease inhibitor mixture tablets (Roche, Mannheim, Germany), rabbit polyclonal anti-CD22 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), horseradish peroxidase-conjugated antiphosphotyrosine Ab (clone 4G10; Upstate Biochemicals, Inc., Lake Placid, NY), FITC, and PE-conjugated antihuman CD22 [clone SHCL-1 (Leu14)] antibody (BD Biosciences, San Diego, CA; PE:antibody ratio, 1:1), FITC-conjugated antihuman CD19 (BD Biosciences), and QuantiBRITE PE-conjugated beads (BD Biosciences).

Cloning of Soluble Human CD22 ECD. The extracellular domain of human β CD22 (amino acids 1–687) was amplified from a human spleen Quick Clone cDNA library (Clontech, Palo Alto, CA) using standard PCR procedure. The PCR primers contained the consensus Kozak sequences (CCACC) and the specific restriction enzyme sites for subsequent cloning linked to the 5′ and 3′ end of the CD22ECD sequences. The expression vector used, pDSRα, was modified from the pSRα vector (21), with a dihydrofolate reductase cassette inserted downstream to the expression cassette driven by the SV40 early promoter.

Expression and Purification of CD22 ECD in Mammalian Cells. Chinese hamster ovary cells (CHOd-) defective in dihydrofolate reductase, originally obtained from L. Chasin (Columbia University, New York, NY) were grown in complete medium (high-glucose DMEM supplemented with 5% FBS, 1% nonessential amino acids, 1% hypoxanthine-thymidine, and 1% glutamine-penicillin-streptomycin).

CHOd− cells were transfected with 10 μg/dish of linearized DNA [pDSRα CD22 ECD (containing amino acids 1–687 of human CD22)] using the calcium phosphate method (Invitrogen). CD22 ECD expression was analyzed by Western blot of serum-free conditioned medium harvested from confluent 24-well cultures. Gels were run under reducing conditions, and blots were probed with a rabbit polyclonal anti-CD22 antibody (Santa Cruz Biotechnology, Inc.). Clones showing the highest expression were expanded and seeded in roller bottles for the production of CD22 ECD.

CD22 ECD was purified from CHO cell-conditioned medium. The concentrated medium was buffer exchanged to 30 mM Tris-HCl, pH 8.5, and applied to a Q-Sepharose column equilibrated in the same buffer. The column was eluted with a linear gradient from 0 to 1 M NaCl in 30 mM Tris-HCl, pH 8.5. Fractions containing CD22 ECD were pooled and applied to a hydroxyapatite column equilibrated in 150 mM NaCl-30 mM Tris-HCl, pH 8.5. Bound protein was eluted with a linear gradient from 0 to 100 mM sodium phosphate, pH 7.0, in 150 mM NaCl. The fractions containing CD22 ECD were pooled and dialyzed into PBS. The final protein concentration was determined by UV absorbance at 280 nm, using an extinction coefficient of 144,270 M^{-1} cm^{-1}. The sample was determined to be ≥98% pure by SDS-PAGE.

Biacore Measurement of Epratuzumab Binding Affinity. Both kinetic and equilibrium analyses of epratuzumab were performed on a BIAcore 3000 (Biacore, Inc., Piscataway, NJ) with PBS and 0.005% P20 surfactant (BIAcore, Inc.) as running buffer.

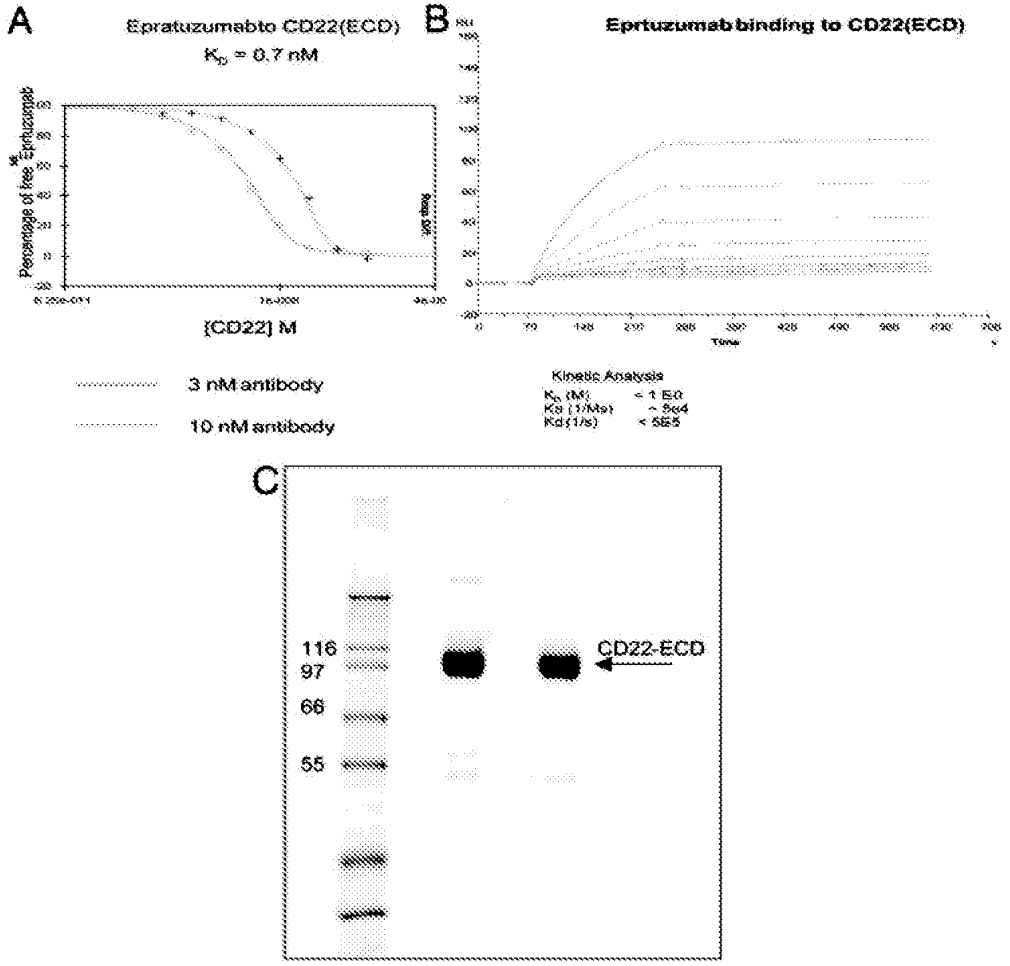

Fig. 1 Epratuzumab measured K_D on Biacore. Affinity of epratuzumab for CD22 ECD was determined by measuring binding of free epratuzumab to a Biacore column after competition with soluble CD22 ECD (*A*). B, kinetics of direct epratuzumab binding (0.8–200 nM) over a CD22 ECD surface, showing a very slow off rate. Cloned, Chinese hamster ovary-expressed, and purified CD22 ECD (as shown in panel *C*) on the blotted membrane with rabbit anti-CD22 was used for molecular characterization of epratuzumab binding.

To study the kinetics of epratuzumab/CD22 binding, CD22 ECD was immobilized to a research grade CM5 sensor chip (Biacore, Inc.) via primary amine groups using the Amine Coupling Kit (Biacore, Inc.) according to the manufacturer's suggested protocol. Resonance level was ~400 Ru. The analysis was effected by injection of increasing concentrations (0.78–200 nM) of epratuzumab over the CD22 surface at a flow rate of 50 μl/min for 3 min, and the dissociation reaction allowed for 15 min. Antibody binding kinetic parameters including k_a (association rate constant), k_d (dissociation rate constant), and K_D (dissociation equilibrium constant) were estimated using the BIA evaluation 3.1 computer program (BIAcore, Inc.).

To analyze the equilibrium constants of epratuzumab-CD22 binding, CD22 ECD was immobilized to a CM5 chip (Resonance level = 2300 Ru) according to the manufacturer's suggested protocol. Epratuzumab at two different concentrations (3 nM and 10 nM) was incubated with increasing concentrations (0.04 nM to 80 nM) of CD22 in sample buffer (PBS + 0.005% P-20 + 0.1 mg/ml BSA) for >2 h to allow samples to reach equilibrium. Samples were then injected over the CD22 surface at 10 μl/min for 30 min. In this system, the binding signal obtained is proportional to the free antibody in solution at equilibrium. The dissociation equilibrium constant (K_D) was obtained from nonlinear regression analysis of the competition curves using a dual-curve one-site homogeneous binding model (KinExA software; Sapidyne Instruments, Inc., Boise, ID).

CD22 Immunoprecipitation and Western Blot Analysis. A total of 10^7 Daudi or Ramos cells (in 15 ml of RPMI 1640 plus 10% FBS) were treated with epratuzumab (5 μg/ml); epratuzumab (5 μg/ml) plus a cross-linking antibody (antihuman IgG(Fc) or $F(ab')_2$ fragments at 5 μg/ml[or antihuman IgM $F(ab')_2$ fragments at 5 μg/ml at 37°C for 15 min. Cells without antibody treatment were used as a negative control. After two washings with cold PBS, cells were centrifuged at 200 × *g* at 4°C for 8 min and resuspended in 400 μl of lysis buffer (1% Triton X-100, 0.1% SDS in 1× PBS with 2 mM sodium orthovanadate and protease inhibitor mixture). Cells were sonicated, kept on ice for 30 min, and centrifuged at 16,000 × *g* at 4°C for 10 min. Epratuzumab (3 μg) and 20 μl of protein G^+/protein A-agarose beads (Oncogen Research Products, Boston, MA) were added to each supernatant and agitated overnight. Beads were then washed three times with lysis buffer. The immunoprecipitated proteins were size fractionated on SDS-PAGE and transferred to nitrocellulose. Membranes were analyzed by immunoblotting with horseradish peroxidase-conjugated antiphosphotyrosine antibody, treated with

stripping buffer (Pierce, Rockford, IL), and reprobed with a rabbit polyclonal anti-CD22.

Primary B Cells from Fresh Tonsils and CLL Samples. Tonsils were collected according to standard surgical procedures, including obtaining patient informed consent, and were shipped overnight in RPMI containing penicillin and streptomycin. Cells were gently mechanically dissociated in cold Hanks' balanced salt solution (Invitrogen), and mononuclear cells were purified using Ficoll-Hypaque (Amersham Biosciences) according to specification. Mononuclear cells were resuspended in Hanks' medium and washed with RPMI containing 10% FBS. Whenever the B cell:total cell ratio fell below 60%, further purification was performed using a T cell depletion kit from Dynal Biotech (Oslo, Norway).

Fresh CLL blood samples were collected in heparinized tubes from patients who had provided informed consent and were shipped overnight on ice. Ficoll-Hypaque purification of mononuclear cells was then performed as previously described. Cells were then immunostained with anti-CD19, anti-CD20, or anti-CD22 as described for FACS analysis and receptor quantification.

Confocal Microscopy Study of Direct Epratuzumab Internalization. FITC-labeled epratuzumab (2 μg/ml) was used to label Daudi cells (10^6 cells/ml) in PBS plus 0.5% BSA, on ice for 30 min. Cells were subsequently washed twice in PBS plus 0.5% BSA, plated in four-well chamber coverslips (Nalge Nunc International), and allowed to incubate for an additional 60 min at either 4°C or 37°C. Confocal images were recorded using an ACAS Ultima confocal microscope (Meridian Instruments, Inc., Okemos, MI) and represent 1-μm sections through the center of a focal plane using a 100× oil immersion objective.

CD22, CD20, and CD19 Receptor Density Quantification. Burkitt's lymphoma cell lines, enriched mononuclear cells from freshly dissociated tonsils, CLL, or normal healthy volunteer peripheral blood samples were used. One million cells per sample were blocked for nonspecific staining with 2% FBS + 1% human serum in PBS (0.05% sodium azide) for 20 min on ice. One microgram of anti-CD19-PE (clone SJ25C1), anti-CD20-PE (clone L27), or anti-CD22-PE [clone S-HCL-1 (Leu-14)] monoclonal antibody with the appropriate isotype controls was added for 30 min at 4°C in the dark. All antibodies were from BD Biosciences. Samples were washed with 2 ml of HBSS and centrifuged at 500 × *g* for 8 min at 10°C. The supernatants were discarded, and the cells were resuspended in 0.5 ml of FACS Lysing Solution (BD Biosciences) and immediately analyzed by flow cytometry (BD FACSCalibur). The CD19, CD20, and CD22 receptor numbers were converted from the standard fluorescence curve set by the QuantiBRITE PE fluorescence quantitation kit (BD Biosciences).

Results

Epratuzumab Binds to CD22 ECD on Biacore with a Measured Affinity of K_D = 0.7 nM. The binding activity of epratuzumab to CD22 ECD was measured on Biacore. In this system, in which CD22 ECD is immobilized, epratuzumab shows a relatively slow off rate ($K_{off} < 10^{-5}$ 1/s; Fig. 1*B*), leading to a K_D of 0.7 nM (Fig. 1*A*) as determined by equilibrium analysis. Although the on rate (k_a) for epratuzumab is relatively slow, the resulting K_D is highly comparable with the reported 8 nM for rituximab (rituximab package insert).

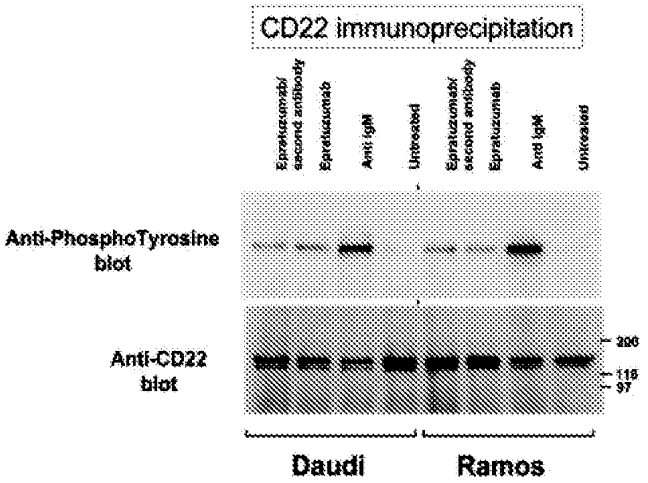

Fig 2 Epratuzumab induces phosphorylation of CD22. Daudi or Ramos cell lines were treated for 15 min at 37°C with a saturating dose of epratuzumab, epratuzumab and a cross-linker, or a B cell receptor activator, anti-IgM. Cells were lysed and CD22 was immunoprecipitated to examine the signaling potential of epratuzumab, using phosphotyrosine-specific antibody (*top*). To ensure equal CD22 loading, immunoblots were stripped and reprobed with a polyclonal rabbit anti-CD22 (*bottom*) (see "Materials and Methods").

Epratuzumab Binding Results in CD22 Phosphorylation. CD22 ligation has been reported by others to induce phosphorylation on its intracytoplasmic tail (10). Tyrosine phosphorylation of the CD22 cytoplasmic tail is also induced by BCR cross-linking. We therefore attempted (*a*) to detect whether epratuzumab could induce CD22 signaling and (*b*) to compare it with the CD22 tyrosine phosphorylation induced by B cell activation, using anti-IgM stimulation (Fig. 2).

Daudi and Ramos Burkitt's lymphoma cell lines were treated for 15 min with saturating concentrations of epratuzumab. Immunoprecipitation-Western blot showed a significant increase in CD22 phosphorylation in both cell lines. Cross-linking epratuzumab using a secondary antibody did not result in higher level of phosphorylation. As expected, stimulation of the BCR using an activating anti-IgM antibody resulted in the highest CD22 phosphorylation level. Epratuzumab binding to tonsil-derived primary B cells also resulted in CD22 phosphorylation above resting level. However, the CD22 phosphorylation level in unstimulated tonsil cells was higher than in the unstimulated cell lines, decreasing the signal:noise ratio (data not shown).

Epratuzumab Internalization Visualized by Confocal Microscopy. In an effort to visually document the rapid, direct internalization of epratuzumab after binding to CD22, epratuzumab was directly labeled with FITC, and its binding to Daudi Burkitt lymphoma cells was monitored at 37°C during 60 min. As a control, Daudi cells were exposed to the same epratuzumab concentration but kept on ice to prevent internalization (Fig. 3, *left*). FITC-epratuzumab/CD22 internalization is evident from capping phenomena and punctate staining of endocytic vesicles (Fig. 3, *arrows*) visible in cells incubated at 37°C only.

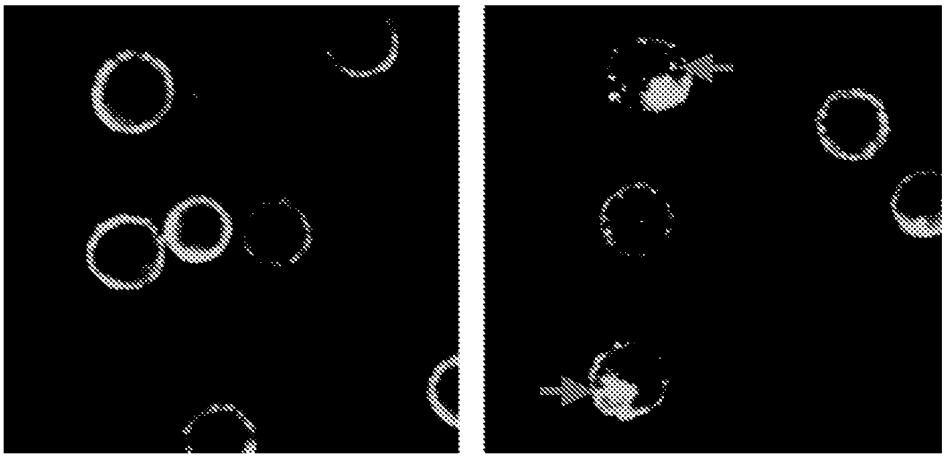

Fig. 3 FITC-epratuzumab direct internalization. FITC-labeled epratuzumab was used to label Daudi tumor cells on ice for 30 min. Cells were subsequently washed and allowed to incubate for an additional 60 min at either 4°C (*left*) or 37°C (*right*). CD22 internalization is evident from capping and punctate staining of endocytic vesicles (*arrows*) visible in cells incubated at 37°C.

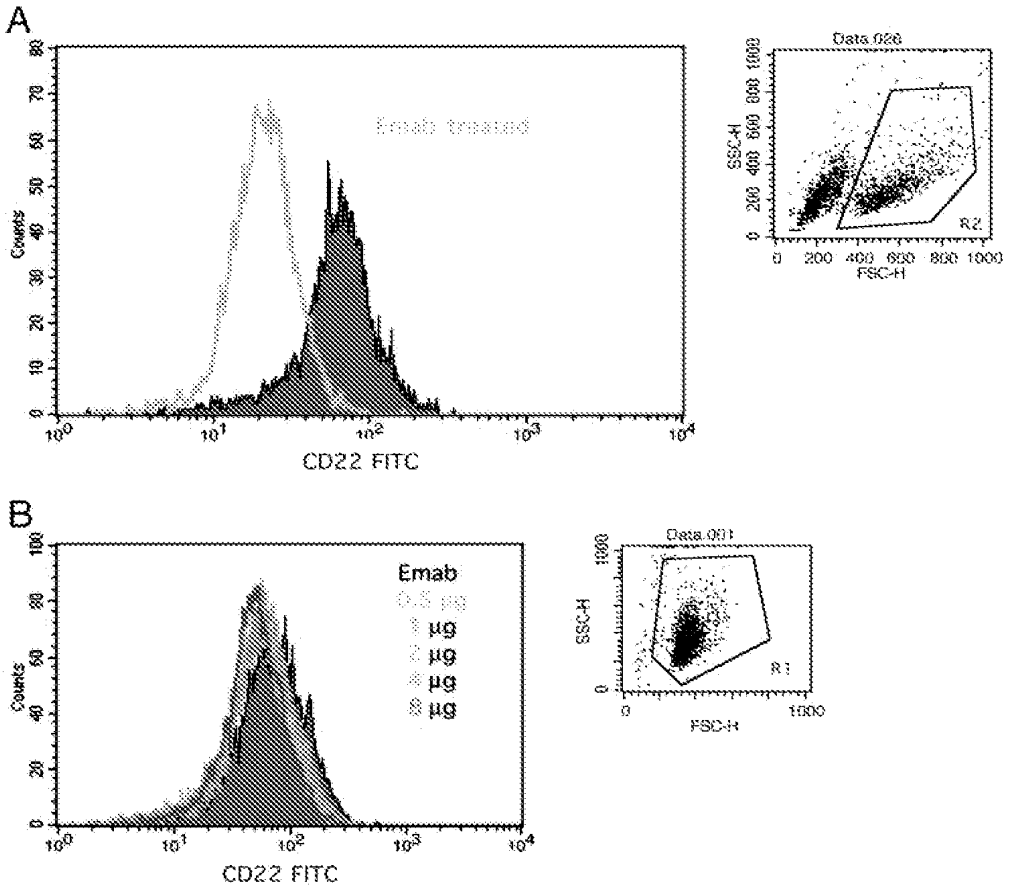

Fig. 4 CD22 internalization as measured by flow cytometry using a noncompeting anti-CD22 antibody. Daudi cells were incubated in the presence of 5 µg/ml epratuzumab (*Emab*) for 20 h at 37°C and analyzed by flow cytometry for CD22 internalization (*A*) as observed by an apparent lowering of cell surface CD22, measured with the FITC-S-HCL1 anti-CD22 monoclonal antibody. B, evidence that adding increasing epratuzumab concentrations together with FITC-labeled S-HCL1 did not result in lowering the signal observed with FITCH-SCI1 alone (*solid blue trace*). This confirms that these two anti-CD22 monoclonal antibodies recognize distinct and noncompeting epitopes on CD22 (FACS using the Daudi cell line). *FSC-H*, forward scatter; *SSC-H*, side scatter.

Internalization of Epratuzumab Results in Quantifiable CD22 Cell Surface Decrease. One particularly intriguing property of CD22 is its capacity to rapidly translocate from the cytoplasm to the cell surface on B cell stimulation on cell lines, (22) and to rapidly internalize upon antibody ligation (23). The result of such internalization is an apparent reduction in the number of cell surface CD22 binding sites (Fig. 4*A*), measurable using a noncompeting anti-CD22 (anti-CD22-PE [clone S-HCL-1 (Leu-14)] monoclonal antibody (24).

We confirmed that the anti-CD22 [clone S-HCL-1 (Leu-14)] monoclonal antibody did not compete with epratuzumab for CD22 binding and therefore could be used as a tool to independently measure cell surface CD22. As shown in Fig. 4*B*, increasing concentration of epratuzumab added together with S-HCL1-FITC labeled antibody did not result in a displacement of the signal observed with S-HCL1-FITC antibody alone.

To quantify cell surface CD22, the S-HCL1 antibody monovalently labeled with PE (conjugated by BD Biosciences)

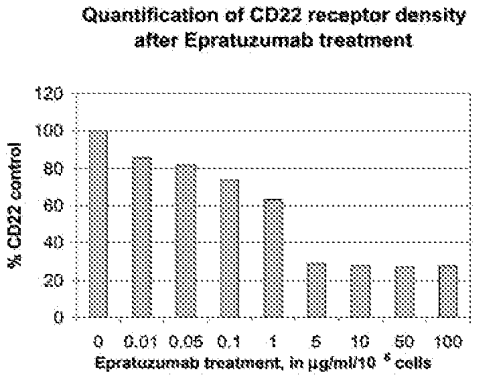

Fig. 5 Quantification of CD22 receptor density after epratuzumab treatment. Daudi lymphoma cells (10^6 cells/ml) were incubated with increasing concentrations of epratuzumab in RPMI supplemented with 10% FBS for 1 h at 37°C. Cells were then washed with cold PBS and immediately analyzed by FACS for CD22 receptor quantification as described. The resulting CD22 density as measured with QuantiBRITE beads and H-SCL1 PE labeled is shown as a percentage of total CD22 on untreated cells (100%). At 5 μg/ml, cells have reached the maximum level of internalization, which is 70% in this experiment.

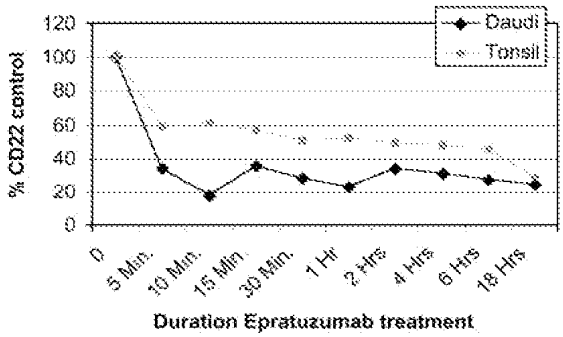

Fig. 6 Epratuzuma-induced CD22 internalization kinetics. Daudi cells and tonsil-derived mononuclear cells were incubated with increasing incubation time in the presence of 25 μg/ml epratuzumab/ At the end of each time point, cells were immunostained with anti-CD22 H-SCL1-PE only (Daudi) or anti-CD19-FITC and anti-CD22-PE (tonsils) for receptor quantification, as described. At early time points, the Daudi cell line is more efficacious in receptor internalization. After prolonged treatment, however (>18 h), no internalization efficiency difference between cell lines and primary cells could be observed.

was then used after cells were incubated with epratuzumab. This allowed indirect measurement of the CD22 receptor internalization rate and extent after a variety of treatments, without direct modification of epratuzumab.

Incubation of Daudi cells for 1 hour with epratuzumab resulted in a dose dependent increase in CD22 internalization (Fig. 5). The point of saturation was reached between 1 to 5 μg/ml/10^6 cells (31.5 nM). At this time point, 30% of the original CD22 density (or 70% internalization) was observed. Similar results were obtained with other cell lines, such as Ramos, Raji, and Namalwa (data not shown), suggesting that the lack of total internalization may reflect more the endogenous turnover of the receptor (25) rather than expression of another CD22 isoform (*i.e.,* the α isoform of CD22, lacking domains 3 and 4 and therefore presumably not bound by epratuzumab).

Consistent with a dynamic equilibrium between rapid epratuzumab-induced CD22 internalization and trafficking to the cell surface of new receptor, prolonged incubation time with epratuzumab resulted in higher degree of internalization, reaching close to 80% after an overnight incubation, in both cell lines (Daudi) and primary tonsil-derived B cells (Fig. 6). At early time points, the Daudi cell line internalizes CD22 very rapidly, consistent with results reported previously for the Raji cell line (23). Internalization of the epratuzumab/CD22 complex on tonsil-derived primary B cells appears much slower. However, the extent and trend for increasing internalization over time did not differ significantly between the two cell populations after a 30-min incubation. To determine whether these observations could be extended to "fresh" lymphoma cells, mononuclear cells isolated by Ficoll-Hypaque from peripheral blood samples from patients with CLL were incubated with saturating concentrations of epratuzumab for 2 h, and CD22 expression was quantified at the end of the incubation time using the QuantiBRITE system (Table 1). Among the CLL and NHL samples tested, CD22 receptor density tends to be lower than in established lymphoma cell lines. However, even in these samples, evidence of CD22 internalization induced by epratuzumab was obtained after a 2-h incubation. For comparison, we tested in the same experiment the B lymphoma cell line Namalwa that expresses low levels of cell surface CD22. Namalwa cells, like Daudi cells, show a very fast and almost maximum level of internalization within minutes (data not shown), indicating that epratuzumab-mediated receptor internalization can occur very efficiently even with low receptor density, but the kinetics may differ significantly between cell lines and primary samples.

CD22 Reexpression after Epratuzumab Treatment. Because in the clinical setting patients will be exposed to epratuzumab for days ($t_{1/2}$ = 23 days), the effect of longer epratuzumab exposure was also explored on Daudi and Ramos cell lines by growing them in the presence of 10 μg/ml epratuzumab for up to 13 days. At each passage/feeding time, cells were counted, and viability was evaluated with trypan blue. No significant difference in cell density and viability could be detected between epratuzumab-treated or control cells (data not shown), suggesting that epratuzumab does not have a direct cytotoxic or cytostatic effect *in vitro*. At day 4 or 7 during the 13-day period, some cells were washed and transferred to non-epratuzumab-containing medium and cultured for an additional 9 or 6 days, respectively. On the last day, all groups of cells were stained with FITC-H-SCL1 anti-CD22 monoclonal antibody and analyzed by flow cytometry (Fig. 7). As shown in Fig. 7, prolonged exposure of cells to epratuzumab for up to 13 days did not result in total abrogation of CD22 cell surface staining. Surprisingly, both Daudi (Fig. 7, *top*) and Ramos (Fig. 7, *bottom*) cell lines did not show full recovery of CD22 expression even after a prolonged (6 and 9 days) period of recovery from epratuzumab treatment when compared with cells grown in control conditions without epratuzumab.

Discussion

CD22 is an appealing target for the development of novel treatments for B cell malignancies because of its expression, which is B cell restricted and developmentally regulated, and its

Table 1 CD22, CD20, and CD19 receptor density, and epratuzumab-induced CD22 internalization on NHL and tonsil primary B cells-derived compared to two Burkitt lymphoma cell lines

Patient	Samples	Receptor density			% internalization
		CD22	CD19	CD20	
1	CLL	1,037	ND[b]	ND	50
2	CLL	2,077	4,770	4,230	40
3	NHL	1,797	3,859	6,758	ND
4	Mantle cell lymphomas	16,907	ND	ND	65
5	CLL	6,107	ND	ND	48
6	Normal tonsils	11,326	3,482	12,485	40–50
7	Normal tonsils	5,570	3,438	40,930	40–50
8	Normal tonsils	6,574	8,201	15,584	40–50
9	Healthy donor	20,047	ND	29,312	ND
	Daudi	22,548	21,787	25,604	60
	Nawalma	5,187	6,309	3,584	50

[a] Primary B cells were enriched from freshly dissociated tonsils (sample 6–8) or blood collected from patients with B-cell malignancies (sample 1–5) or healthy volunteer (sample 9). Cells were immediately analyzed for receptor density quantification as described, or treated with saturating dose of epratuzumab, for 2 hours at 37°C.

[b] ND, not done.

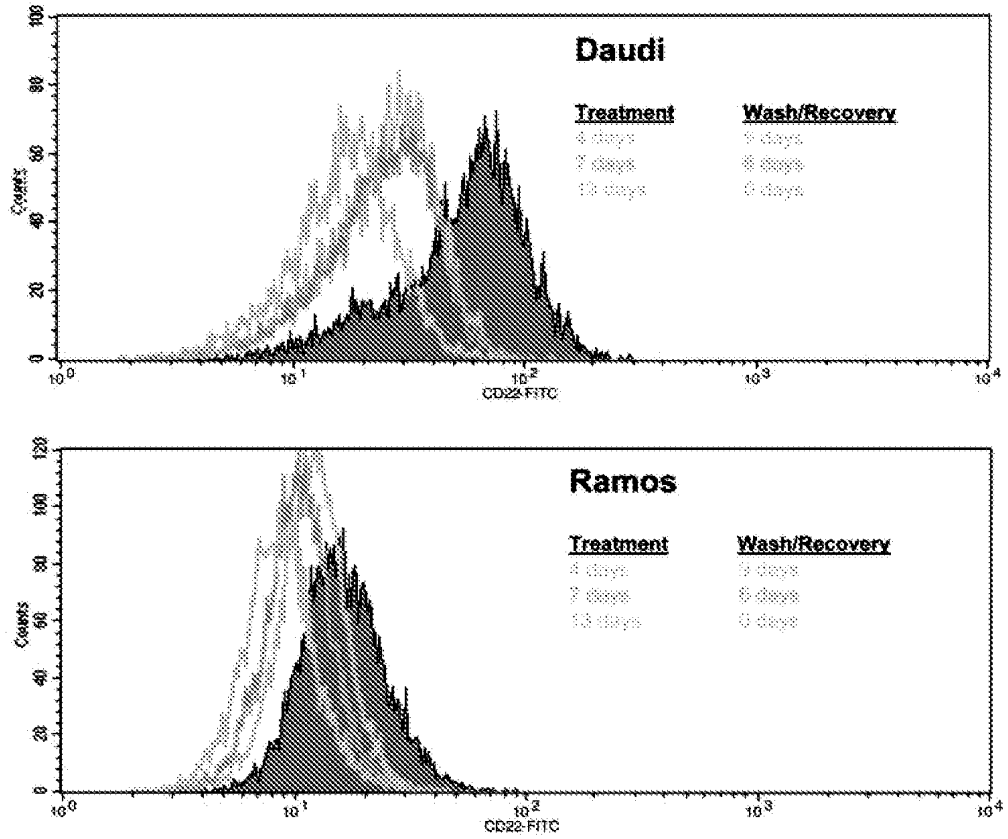

Fig. 7 Partial recovery of surface CD22 after epratuzumab treatment. Daudi or Ramos cell lines were grown in RPMI-10% FBS with added 20 μg/ml epratuzumab (or no added antibody for negative control) for up to 13 days. After 4 or 7 days treatment, cells were washed and allowed to recover in non-antibody-containing growth medium for 6–9 days. In all, the experiment lasted for 13 days, during which cell density was kept at 10^6 cells/ml, and viability was >95%. At the end of 13 days, all groups were analyzed by flow cytometry for CD22 expression using H-SCL1-FITC labeled (BD Biosciences), and the profiles were overlaid with that of untreated control cells (*solid blue trace*).

physiological functions as an adhesion molecule and a regulator of B cell receptor activation. One of the main functions of CD22 is to regulate B cell responses through recruitment of key signaling molecules to the antigen/receptor complex. Part of the BCR activation pathway includes tyrosine phosphorylation of the CD22 intracellular tail. However, CD22 ligand-mediated signaling can also be achieved by engaging the CD22 ligand binding site (mapped to CD22, domains 1 and 2) (5).

Epratuzumab is a humanized monoclonal antibody directed against the CD22 molecule; the parental murine antibody (LL2) was originally developed by Goldenberg *et al.* by "classic" immunization of mice with Raji cells as a source of B cell

antigens. This report confirms unequivocally that epratuzumab binds to CD22 ECD. The epratuzumab K_D value measured in this study on Biacore is consistent with previously published data on epratuzumab binding affinity to Raji cells using radiolabeled LL2 competed out with cold LL2 (estimated K_D from these experiments would be in the low nM range) (26). This compares favorably with other monoclonal antibodies used in the treatment of B cell lymphomas; for instance, rituximab affinity is reported to be 8 nM (rituximab package insert).

Epratuzumab binding to CD22 does not block the ligand-binding site on CD22 (17). In fact, as opposed to the ligand blocking anti-CD22 monoclonal antibodies (10), epratuzumab did not show evidence of inducing apoptosis or growth arrest on lymphoma cell growth *in vitro* (data not shown). Because CD22 interacts with a variety of ligands (including CD45) and cellular partners (including T cells) (5, 27), the effect of modulating this receptor should be significant *in vivo* but might be difficult to demonstrate in an isolated context *in vitro*. For example, others have shown that blocking CD22 translocation by using beads coated with anti-CD22 antibodies can alter the B cell threshold of activation thorough BCR (28).

In addition to its signaling regulation of B cell activation, CD22 was shown by several groups to internalize rapidly on antibody ligation (29). In the majority of cases internalization was demonstrated using radiolabeled antibody. In this report, we confirm the results previously published by using FITC-labeled epratuzumab as well as by measuring "naked" epratuzumab binding/internalization indirectly. In particular, in our system using confocal microscopy the internalization of the epratuzumab/CD22 complex could be visualized as intracytoplasmic vesicles presumably of endosome-lysosome origin. The internalization was dose dependent and long lasting, without reaching reversibility at clinically meaningful concentrations. Moreover, epratuzumab prolonged incubation results, at a given dose, in a progressive decrease in CD22 surface level, presumably shifting the equilibrium between internalization and receptor turnover. However, total disappearance of CD22 from the cell surface was never observed. These results could be explained by the known coexpression of a shorter spliced variant of CD22, anti-CD22. This spliced mRNA minor variant found on human B cells is missing exons 5–8, resulting in CD22 ECD that lack domains 3 and 4 (30). This isoform therefore would not bear the epitope to which epratuzumab binds. However, using cell lines known to express various ratios of the two isoforms, similar apparent incomplete depletion of cell surface CD22 (data not shown) after prolonged epratuzumab incubation was observed, thus making the above hypothesis unlikely. Because in the clinical setting patients will be exposed to epratuzumab for a long period of time, we are now measuring the effect of epratuzumab treatment on CD22 mRNA expression as well as on time for complete recovery of cell surface CD22.[4] Reexpression of CD22 after prolonged epratuzumab treatment appears to follow slow kinetics; this was determined on two B cell lines grown for a different number of days in antibody-free medium after being exposed to epratuzumab for various periods of time. To our surprise, even after cells were washed and grown for 4–9 more days in what should be essentially epratuzumab-free medium, the CD22 level did not fully recover compared with untreated cells. It is possible that the actual epratuzumab concentration in fresh medium is modified by the epratuzumab pool bound to cytoplasmic CD22 being released during cell division or that CD22 expression at the RNA level is down-regulated by the antibody treatment. This is an important point to address in future experiments, because it may have implications in epratuzumab dosing/scheduling in the clinic.

In conclusion, the CD22 molecule serves at least two important roles, *i.e.*, regulation of BCR activation (mostly negative) and involvement in mature B cell homing. Both are key functions that epratuzumab can modulate through induction of CD22 phosphorylation and CD22 internalization, respectively. Although difficult to demonstrate *in vitro*, these effects could have very different implications depending on the stage of maturation of B cells, and their compartment localization. For example, recent reports suggest adhesion molecules to be implicated in homing, dissemination, and survival of NHL cells (31, 32).

Overall the results presented here, although not directly answering the question of the *in vivo* mechanism of action of epratuzumab, do help to clearly differentiate this antibody from other monoclonal antibodies currently used in the treatment of NHL. This differentiation, at least theoretically, forms the basis for the clinical evaluation of combination therapies.

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